Interactions of the major effective components in Shengmai formula with breast cancer resistance protein at the cellular and vesicular levels

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ABSTRACT

Shengmai formula is one of the traditional Chinese medicine representative formulas and is widely used for the treatment of cardio- and cerebrovascular disease. Previous studies demonstrated that the major effective ingredients in SMF can interact with each other based on some uptake transporters. However, the role of the efflux transporter breast cancer resistance protein (BCRP) in these interactions involving SMF remains unclear. The purpose of this study was to investigate the interactions of the major active components of SMF with BCRP and the compatibility mechanism of these complex components in SMF based on BCRP. We selected 4 main fractions, including ginseng total saponins (GTS), ophiopogon total saponins (OTS), ophiopogon total flavonoids (OTF), and fructus schisandrae total lignans (STL), and 12 bioactive components, including ginsenosides Re, Rd, Rb1, and Rg1, ophiopogonins D and D′, methylophiopogonanones A and B, schizandrins A and B, and schizandrols A and B to explore the interactions of SMF with BCRP in LLC-PK1 and LLC-PK1/BCRP cells and BCRP membrane vesicles. The results showed that ginsenosides Re and Rg1, methylophiopogonanone B, and schizandrin A can be transported by BCRP into LLC-PK1/BCRP cells. Schisandrol B exhibited a markedly inhibitory effect on the transport function of BCRP and can significantly inhibit the uptake of methylophiopogonanone B and schizandrin A into LLC-PK1/BCRP cells. In "Inside-Out" BCRP membrane vesicles, BCRP mediated the transport of ginsenosides Re and Rg1, methylophiopogonanone B, and schizandrin A, with K_m values of 111.9 ±31.26 μM, 82.01 ±16.72 μM, 57.06 ±8.789 μM, and 37.19 ±6.512 μM, respectively. GTS, STL, ginsenosides Rd and Rb1, and schisandrol B were potent inhibitors of BCRP and showed different degrees of inhibition on the transport of ginsenosides Re and Rg1, methylophiopogonanone B, and schizandrin A *via* BCRP. In conclusion, GTS, STL, ginsenosides Rd and Rb1, and schizandrol B are potential inhibitors of BCRP. Ginsenosides Re and Rg1, methylophiopogonanone B, and schizandrin A are potential substrates of BCRP, and their transport, which is mediated by BCRP, may be inhibited by potential inhibitors in SMF. There are potential interactions of these main effective components of SMF at the cellular and vesicular levels that are mediated by BCRP. The interplay of these bioactive components based on BCRP may be an important compatibility mechanism in SMF.

1. Introduction

Traditional Chinese medicine (TCM) combined with synthetic drugs is widely used in East Asian countries. In addition, international communities have placed increasing emphasis on the use of TCM based on its unique philosophy and therapeutic model [1]. However, the interactions between TCM and synthetic drugs, as well as among the active ingredients in TCM, are often harmful to patients [2,3]. Investigations have shown that many compounds in TCM often interact with each other, resulting in changes in pharmacokinetic profiles [4] and pharmacological effects [5]. For example, the combinations of Sophorae flavescentis radix (Kushen) and glycyrrhizae radix et rhizoma (Gancao) can cause pharmacokinetic interactions by affecting the activity of CYP enzymes [6].

Drug transport is also one of the crucial means of drug disposal *in vivo* and mediates various interactions between TCM and drugs, particularly in the liver [7,8]. For instance, quercetin inhibits OATP1B1-mediated uptake of pravastatin *in*

Abbreviations: SMF, Shengmai formula; BCRP, breast cancer resistance protein; GTS, ginseng total saponins; OTS, ophiopogon total saponins; OTF, ophiopogon total flavonoids; STL, schisandrae total lignans; AUC, area under the curve; C_{max}, maximum concentration; MTX, methotrexate; IC_{50}, half-maximal inhibitory concentration; K_m, Michaelis constant; V_{max}, maximum velocity; LC–MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization; ERs, efflux ratios.

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vitro and has a modest inhibitory influence on the pharmacokinetics of pravastatin in healthy volunteers [9]. The breast cancer resistance protein (BCRP) is one of the most important efflux proteins that modulates the pharmacokinetics of many drugs, nutrients, and toxicokinetics of toxicants and often interacts with compounds in TCM [10–12]. Karibe et al. [13] found that curcumin pretreatment significantly increases oral AUC, C_{max}, and bioavailability of sulfadiazine and rosuvastatin in cynomolgus monkeys, thereby verifying the curative effect of curcumin on BCRP function in the gastrointestinal tract. Glycyrrhetinic acid increases the accumulation of entecavir at the cellular level and enhances its antiviral activity by inhibiting BCRP [14]. There is currently an increasing number of studies focusing on TCM-drug interactions that are mediated by BCRP.

Shengmai formula (SMF) is composed of *Panax ginseng* C. A. Mey, *Ophiopogon japonicus* (Linn. f.) Ker-Gawl, and *Schisandra chinensis* (Turcz.) Baill with a ratio of 1□3.12□1.56. Based on its capacity to improve myocardial ischemia, cardiac stress, and anti-arrhythmia, SMF has been widely used for the treatment of cardio- and cerebrovascular disease [15]. Our previous studies showed that Shengmai injection increases the plasma concentration of ophiopogonin D in rats compared to the same dose of ophiopogonin D alone [16]. Ginsenoside Rb1 and Rd in Shengmai injection significantly inhibit liver ophiopogonin D uptake, which is mediated by OATP1B1/1B3 [17]. NTCP also mediates the interactions of the main components in SMF in NTCP-HEK293 T cells. These results reveal the potential interactions between the major active ingredients in SMF and uptake transporters. Additionally, Liang et al. [18] found that Schisandra lignans extract (SLE) has a significant inhibitory effect on P-gp, and confirmed that SLE can increase the exposure of ginsenoside Rb2, Rc, Rg2, Rg3, Rd, and Rb1 by inhibiting P-gp *in vivo* and *in vitro* [19]. Jin et al. [20] confirmed the interaction between ginseng and BCRP. However, there is little concern about the interactions between ophiopogonis and schisandrae and BCRP. To date, the role of BCRP in these interactions and the compatibility mechanism of the complex components in SMF remain unclear.

This study selected 4 main fractions, including ginseng total saponins (GTS), ophiopogon total saponins (OTS), ophiopogon total flavonoids (OTF), and fructus schisandrae total lignans (STL), and 12 bioactive components, including ginsenoside Re, Rd, Rb1, and Rg1, ophiopogonins D and D´, methylophiopogonanones A and B, schizandrins A and B, and schizandrols A and B to explore the interactions of SMF with the efflux transporter BCRP at the cellular and vesicular levels. We paired the potential substrates and inhibitors with each other and illustrated the compatibility mechanism of the complex components in SMF based on BCRP. **2. Materials and methods**

2.1. Chemicals and reagents

Ginsenoside Re, Rd, Rb1, and Rg1 were purchased from Macklin Biochemistry Technology (Shanghai, China). Methylophiopogonanones A and B, as well as ophiopogonins D and D´, were purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Schizandrins A and B, schizandrols A and B, and methotrexate were provided by the National Institutes for Food and Drug Control (Beijing, China). The purity of the above compounds was > 98 %. GTS (including 362.08 μg, 36.42 μg, 27.18 μg, and 5.46 μg of ginsenoside Rb1, Rd, Rg1, and Re per milligram, respectively), OTS (including 567.42 μg and 21.75 μg of ophiopogonins D and D´ per milligram, respectively), OTF (including 432.75 μg and 344.08 μg of methylophiopogonanones A and B per milligram, respectively), and STL (including 563.12 μg of schizandrin A, 182.20 μg of schizandrin B, 96.53 μg of schizandrol A, and 56.04 μg of schizandrol B per milligram) were obtained from Qigu Biotechnology (Shanghai, China). The purity of the above four tests was >95 %. Lucifer yellow and ATP were purchased from Sigma-Aldrich (St. Louis, MO, USA). LLC-PK1 and LLC-PK1/BCRP cells were purchased from Shanghai Genechem Co., Ltd. Inside-out membrane vesicles (prepared from insect cells expressing human BCRP) were obtained from Geno Membrane (Kanazawa, Japan).

2.2. Cellular transport assays

2.2.1. Cellular uptake experiments

LLC-PK1 and LLC-PK1/BCRP cells were used in the uptake experiments. The cells were grown in medium 199 supplemented with 10 % FBS and 100 U mL^{-1} penicillin G with 100 μg mL^{-1} streptomycin and maintained at 37 °C in the presence of 5 % CO_2. Before the study, we pre- incubated the LLC-PK1 and LLC-PK1/BCRP cells with warm Hank's balanced salt solution HBSS for 30 min, then discarded HBSS and incubated the cells with the test solution containing MTX (20 μM) or the main components of SMF (10, 50, 100 μM) and BCRP inhibitor Ko143 (10 μM) for 2 h at 37 °C, then discarded all the solutions and added ice- cold water to stop the reaction. Finally, the concentrations of MTX and the main components of SMF were measured using the liquid chromatography-mass spectrometry (LC/MS) method. In our study, all the tested compounds were dissolved in DMSO and the final concentration of DMSO was no more than 0.1 %. All the experiments were conducted in triplicate.

2.2.2. Bidirectional transport experiments

The LLC-PK1 and LLC-PK1/BCRP cells were grown on Transwell® monolayers for seven days, and transmembrane resistance exceeding 200 ohms was required before use in the experiment. First, the cells were washed with warm HBSS twice and pre-incubated with HBSS for 30 min at 37 °C. Then, MTX (20 μM) or the main components of SMF (100 μM) and BCRP inhibitor Ko143 (10 μM) were added to either the apical or basolateral side. Finally, the concentrations of MTX and the main components of SMF were measured using an LC/MS method. All experiments were conducted in triplicate.

2.3. Vesicular transport assays

After pre-incubation for 5 min at 37 °C, the reaction system, including vesicle, test chemicals, 50 mM MOPS-Tris, 70 mM KCl, and 7.5 mM $MgCl_2$ (pH 7), was supplemented with ATP or AMP buffer to start the reaction for 10 min at 37 °C. The reaction was terminated by adding 200 μL of ice-cold "stop and wash buffer" (including 400 mM MOPS-Tris, 700 mM KCl). The solution was immediately transferred to a 96-well MultiScreen filter plate (pore size: 0.65 μm; Millipore, Billerica, MA, USA) and washed five times with "stop and wash buffer". Vesicles were finally solubilized in methanol. Concentrations of lucifer yellow were analyzed by measuring the fluorescence intensity at an excitation wavelength of 430 and emission wavelength of 538 nm. The concentrations of the effective components in SMF transported by BCRP in vesicles were measured using LC/MS.

2.4. LC–MS analysis

The concentrations of ginsenoside Re, Rd, Rb1, and Rg1, methylophiopogonanones A and B, ophiopogonins D and D´, schizandrins B and A, and schizandrols A and B were detected by an LC/MS method with the Shimadzu LC/MS 2010 EV system as previously described [21]. For the detection of ginsenosides Re, Rd, Rb1 and Rg1, the mobile phase consisted of 0.05 mM ammonium chloride and acetonitrile, and digoxin was used as the internal standard. For the determination of methylophiopogonanones A and B, ophiopogonins D and D´, the mobile phase were made up of acetic acid and acetonitrile. Ophiopogonin D was used as the internal standard when analyzing methylophiopogonanones A and B. However, methylophiopogonanone A was used as the internal standard when analyzing ophiopogonins D and D´. For the quantification of schizandrins A and B, schizandrols A and B, the mobile phase contained 0.1 % formic acid and acetonitrile (25:75, v/v), and bicyclol was used as the internal standard. For the analysis of MTX, the mobile phase included 0.1 % formic acid and acetonitrile (55:45, v/v), and metronidazole was used as the internal standard.

2.5. Data processing and statistical analysis

The kinetic parameters K_m, V_{max}, and IC_{50} were estimated from curve fitting using GraphPad Prism 5 software. The permeability of these alkaloids was estimated by calculating the apparent permeability coefficient (*P*app) as follows: *P*app =dQ/dt * A * C_0, where C_0 is the initial drug concentration, A is the surface area of the monolayer equal to 1.12 cm^2, and dQ/dt is the rate of appearance of drugs on the receiver side. Efflux ratio (ER) was calculated as follows: ER = *P*app (B - A)/*P*app (A - B). Data were expressed as the mean ±SE (n =3). The differences between the mean values were analyzed for significance using Student's *t*-test (unpaired, two-tail). Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Uptake and transport of the main components of SMF in LLC-PK1/ BCRP cells

The effect of a BCRP inhibitor (Ko143) on the uptake and transport of 12 bioactive components of SMF was examined in LLC-PK1 and LLC- PK1/BCRP cells to determine which components are potential substrates of BCRP. MTX was used as a positive control in the experiment.

In the uptake study, the intracellular accumulation of these components in LLC-PK1 and LLC-PK1/BCRP cells was evaluated after incubating for 2 h. Compared to the LLC-PK1 cells, the LLC-PK1/BCRP cells showed less cellular accumulation of the BCRP substrate. The effect of various concentration of ko143 (5, 10, 20 μM) on the uptake of MTX (20 μM) was assessed and the results showed that 10 μM of Ko143 could significantly inhibit the efflux function of BCRP (Fig. 1). Finallly, 10 μM of Ko143 was selected in the subsequent study [22]. The application of Ko143 (10 μM) resulted in a significant increase in the uptake of ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A in LLC-PK1/BCRP cells, but did not in LLC-PK1 cells (Fig. 2).

Then a bi-directional transport study was conducted to further verify the results of the cellular accumulation study. Fig. 3 illustrates the efflux ratios (ERs) of the 12 bioactive components of SMF in LLC-PK1 and LLC- PK1/BCRP cells in the absence and presence of 10 μM Ko143. The application of Ko143 caused a significant decrease in ERs of ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A in LLC-PK1/ BCRP cells from 2.23 to 1.67, 2.54 to 1.43, 4.10 to 2.01, and 5.51 to 2.70, respectively (Fig. 3B). The ERs of other ingredients were all < 2 and hardly changed after the addition of Ko143.

3.2. Effects of the main components in SMF on MTX uptake and transport in LLC-PK1/BCRP cells

The influences of the 4 main fractions and the 12 bioactive components in SMF on BCRP-mediated MTX uptake were investigated. Fig. 4A showed that schizandrol B (100 μM) exhibited a significant effect on the uptake of MTX (20 μM) as mediated by BCRP. Schizandrol B (100 μM) and Ko143 (10 μM) both increased the MTX uptake by 2.13-fold and 2.04-fold, respectively.

The effects of the 4 main fractions and the 12 bioactive components in SMF on the ERs of the BCRP substrate in LLC-PK1/BCRP cells were further validated. MTX (20 μM) transport across LLC-PK1/BCRP cell monolayers in the absorptive (AP→BL) and secretory (BL→AP) directions, and the corresponding Papp values are shown in Fig. 4B. MTX, a classic BCRP substrate, exhibited an efflux ratio of 7.89, which was far greater than 2.0. This ER of MTX significantly decreased to 3.91 in the presence of 10 μM Ko143. Schizandrol B at 100 μM also greatly decreased the ER of MTX from 7.89 to 3.83. Further study (Fig. 4C and D) demonstrated that schizandrol B could enhance MTX uptake or reduce the ER of MTX in concentration-dependence from 50 to 100 μM.

3.3. Interactions of the major components in SMF in LLC-PK1/BCRP cells

The above results demonstrated that ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A were the potential substrates of BCRP, and schizandrol B imparted an inhibitory effect on BCRP activity, so the interactions of these components in SMF as mediated by BCRP were further investigated. Fig. 5A shows that schizandrol B enhances the uptake of schizandrin A and methylophiopogonanone B as mediated by BCRP at a concentration of 100 μM. However, the uptake of ginsenoside Re and Rg1 was not affected by 100 μM schizandrol B. The presence of schizandrol B could decrease the ERs of schizandrin A and methylophiopogonanone B from 5.82 to 4.02 and 3.91 to 2.97, respectively. However, the ERs of ginsenoside Re and Rg1 were not affected by

Fig. 2. The cellular accumulation of ginsenoside Re (A), ginsenoside Rg1 (B), methylophiopogonanone B (C), and schizandrin A (D) in LLC-PK1 and LLC-PK1/BCRP cells was evaluated in the presence or absence of Ko143 (10 μM). The data are presented as the mean ±SE of three independent experiments. *p <0.05, **p <0.01, and ***p <0.001.

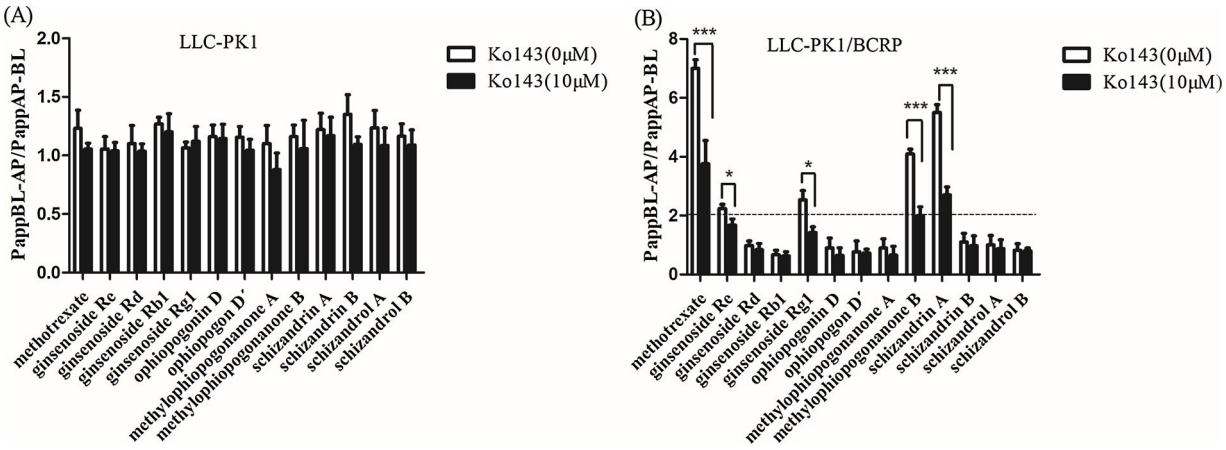


Fig. 3. The efflux ratios (PappBL-AP/PappAP-BL) of MTX (20 μM) and 12 bioactive components of SMF (100 μM) in LLC-PK1 (A) and LLC-PK1/BCRP (B) cells were evaluated in the absence and presence of Ko143 (10 μM). The data are presented as the mean ±SE of three independent experiments. *$p < 0.05$, **$p < 0.01$, and

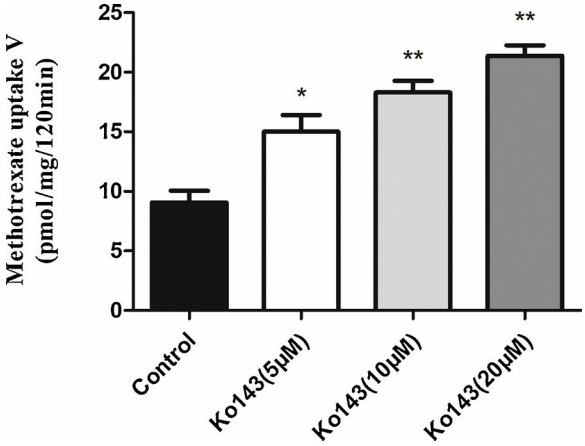


Fig. 1. The effects of various concentration of Ko143 (5, 10, 20 μM) on the uptake of MTX (20 μM) in LLC-PK1/BCRP cells were evaluated. The data are presented as the mean ±SE of three independent experiments. *p <0.05, **p <0.01, and ***p <0.001.

100 μM schizandrol B (Fig. 5B).

3.4. Transport of the main components in SMF in BCRP membrane vesicles

First, we assessed the kinetic parameters of the BCRP classic substrate lucifer yellow. The kinetics were characterized by an apparent affinity (K_m) of 18.86 ±2.149 μM and a maximum rate (V_{max}) of 254.8 ±8.312 pmol/mg protein/min, which was basically consistent with a previous report [23], indicating that this BCRP-expressing vesicle model is available. Then, we selected two concentrations of 10 μM and 100 μM to study whether the 12 bioactive components of SMF were the substrates of BCRP. The results showed that ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A could be transported by BCRP (transport $_{ATP}$/transport $_{AMP}$ ratios were all > 2). Their K_m values (μM) were 111.9 ±31.26, 82.01 ±16.72, 57.06 ±8.789, and 37.19 ±6.512, respectively. Their V_{max} values (pmol/mg protein/min) were 39.37 ±5.197, 40.84 ±3.467, 131.2 ±8.692, and 168.3 ±10.82, respectively (Fig. 6).

***$p < 0.001$.

3.5. Effects of the main components in SMF on lucifer yellow transport in

Fig. 4. The uptake (A) and efflux ratio (B) of MTX (20 μM) in the presence of 4 effective fractions and 12 bioactive components of SMF (100 μM) and the effect of schizandrol B (10, 50, 100 μM) on the uptake (C) and efflux ratio (D) of MTX (20 μM) in LLC-PK1/BCRP cells were evaluated. Ko143 (10 μM), an inhibitor of BCRP, was used as the positive control. The data are presented as the mean ±SE of three independent experiments. *$p <$ 0.05, **$p <$ 0.01, and ***$p <$ 0.001.

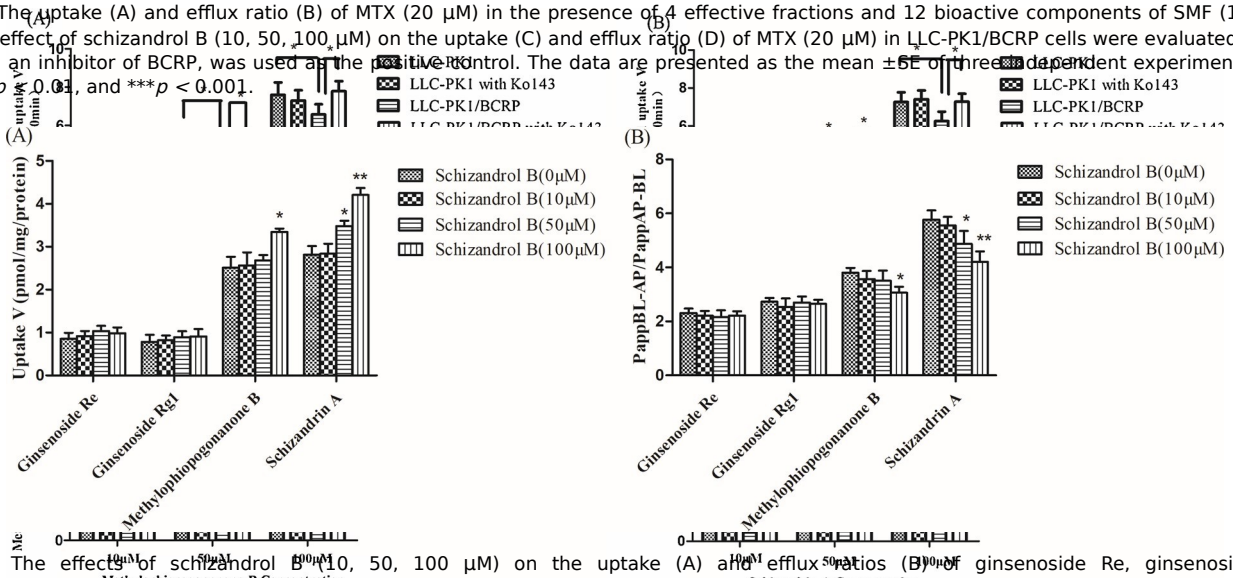


Fig. 5. The effects of schizandrol B (10, 50, 100 μM) on the uptake (A) and efflux ratios (B) of ginsenoside Re, ginsenoside Rg1, methylophiopogonanone B, and schizandrin A (10 μM) in LLC-PK1/BCRP cells were evaluated. The data are presented as the mean ±SE of three independent experiments. *$p <$ 0.05, **$p <$ 0.01, and ***$p <$ 0.001.

BCRP membrane vesicles

Among the four main fractions, GTS and STL exhibited a stronger inhibitory effect on BCRP transport activity. They can inhibit the transport of lucifer yellow by 89 % and 70 % at a concentration of 100 μM. Among the 12 main active ingredients, only ginsenoside Rd and Rb1 and schizandrol B imparted inhibitory effects on the transport activity of BCRP. In the presence of 100 μM ginsenoside Rd and Rb1 and schizandrol B, the intake of lucifer yellow decreased by 78 %, 59 %, and 74 %, respectively. Ko143 at a concentration of 1 μM inhibited the intake of lucifer yellow by 92 %. The IC_{50} values of ginsenoside Rd and Rb1 and schizandrol B were 20.46 ±1.47 μM, 42.02 ±2.31 μM, and 27.13 ±5.49 μM, respectively. Ginsenoside Rd and Rb1 and schizandrol B inhibited the uptake of lucifer yellow as mediated by BCRP through a non-competitive inhibition mode, with V_{max} decreasing from 254.8 ±8.322 to 180 ±4.751 pmol/mg protein/min, from 254.8 ±8.322 to 207.8 ±6.381 pmol/mg protein/min, and from 254.8 ±8.322 to 202.3 ±4.232 pmol/mg protein/min, whereas their K_m values did not change (Fig. 7).

3.6. Interactions of the major components in SMF in BCRP membrane vesicles

The above results of the vesicle study demonstrated that ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A are the potential substrates of BCRP, and GTS, STL, ginsenoside Rd and Rb1, and schizandrol B are the potential inhibitors of BCRP. Then, the interactions of these components in SMF as mediated by BCRP were further explored. The BCRP-mediated transport of ginsenoside Re significantly decreased by 29 %, 21 %, and 17 %, respectively, in the presence of STL, ginsenoside Rd, and schizandrol B at a concentration of 50 μM (p <0.05). However, ginsenoside Rb1 had no significant effect on the transport of ginsenoside Re ($p >$ 0.05). The transport of ginsenoside Rg1, which is mediated by BCRP, was significantly inhibited by 52 %, 30 %, 19 %, and 18 %, respectively, after the addition of 50 μM STL, ginsenoside Rd and

***$p <$ 0.001.

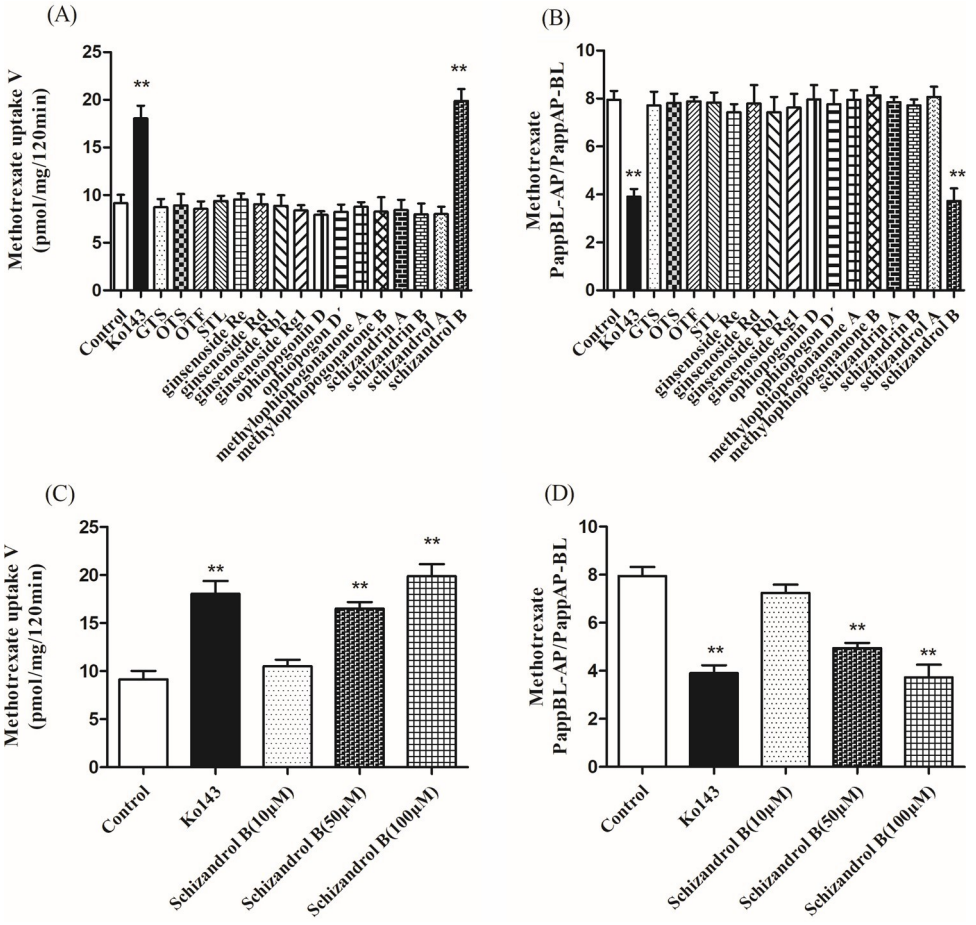
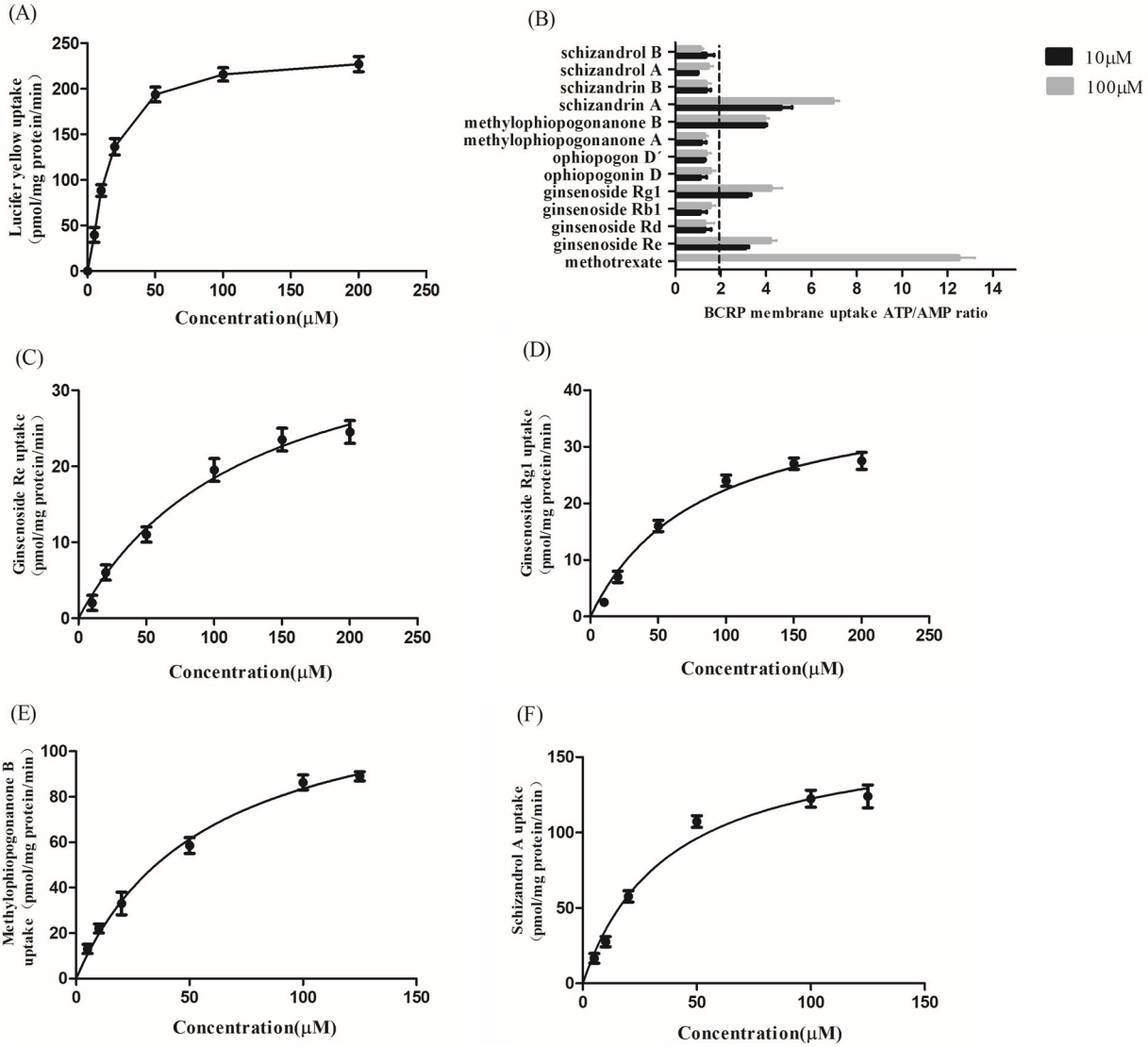


Fig. 6. The concentration-dependent uptake of lucifer yellow (A), ginsenoside Re (C), ginsenoside Rg1 (D), methylophiopogonanone B (E), and schizandrin A (F), and the membrane uptake ATP/AMP ratio in the presence of MTX and 12 bioactive components of SMF (B) were evaluated in BCRP membrane vesicles. Positive vesicular transport as mediated by BCRP was defined as a net transport rate ratio ($Transport_{ATP}$/$Transport_{AMP}$ ratio) > 2. The data are presented as the mean ±SE of three independent experiments.

Rb1, and schizandrol B. GTS, STL, ginsenoside Rd and Rb1, and schizandrol B at a concentration of 50 μM significantly inhibited BCRP- mediated transport of methylophiopogonanone B by 71 %, 55 %, 31 %, 22 %, and 26 %, respectively. The BCRP-mediated transport of schizandrin A was also attenuated by GTS, ginsenoside Rd and Rb1, and schizandrol B at 50 μM, and the uptake decreased by 85 %, 54 %, 33 %, and 32 % (Fig. 8).

4. Discussion

Long-term clinical practice has confirmed the unique compatibility law and therapeutic effects of TCM. Recently, TCM once again showed its important role in the prevention and treatment of COVID-2019 in China [24]. TCM is always thought to have fewer adverse reactions than synthetic drugs. However, herb-drug interactions often cause adverse events. Numerous studies have confirmed that transporters play an important role in herb-drug interactions. SMF is a TCM representative formula that is widely used in clinical practice. The interaction between SMF and the uptake transporters had been previously explored, including OATP1B1, OATP1B3, and NTCP. Nevertheless, the interaction between SMF and BCRP remains unclear. Thus, the drug interactions and compatibility mechanisms in the complex components of SMF mediated by BCRP should be investigated (Fig. 9).



In this study, first, we investigated the effect of the BCRP inhibitor Ko143 on the uptake of 12 main bioactive components of SMF in LLC- PK1 and LLC-PK1/BCRP cells to determine which components are the potential substrates of BCRP. The results showed that Ko143 (10 μM) significantly increased the uptake of ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A in LLC-PK1/BCRP cells, but not in LLC-PK1 cells (Fig. 2). Interestingly, Ko143 can not enhance the uptake of low concentration of ginsenoside Re (10 μM) in LLC-PK1/BCRP cells. It may be explained that there are multiple substrate-binding sites in transporter BCRP, and ginsenoside Re can weakly bind to someone substrate-binding site of BCRP and shows a low affinity for BCRP. Bi-directional transport experiments indicated that the application of Ko143 (10 μM) resulted in a significant decrease in the ERs of ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A (100 μM). And the ERs of these four components were all greater than 2 in LLC-PK1/BCRP cells (Fig. 3). Based on the above results, we hypothesize that ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A are the potential substrates of BCRP. Then, the effects of the main components in SMF on the uptake and transport of MTX (BCRP substrate) were investigated to confirm whether these are potential BCRP inhibitors in LLC-PK1/BCRP cells. The results showed that only schizandrol B significantly increased the uptake of MTX (Fig. 4A) in LLC- PK1/BCRP cells and reduced the ERs of MTX at a concentration of 100 μM in the bidirectional transport experiments (Fig. 4B). Various concentration studies demonstrated that schizandrol B could inhibit the transport of MTX mediated by BCRP in a concentration-dependence manner from 50 to 100 μM and schizandrol B may be a weak inhibitor of BCRP. Based on the above results, the interactions of these components in SMF, which are mediated by BCRP, were further studied. The results showed that schizandrol B has no inhibitory effect on the transport of ginsenoside Re and Rg1, but high concentration of schizandrol B (100 μM) can inhibit the transport of methylophiopogonanone B and schizandrin A, which may be due to the different affinity of the four

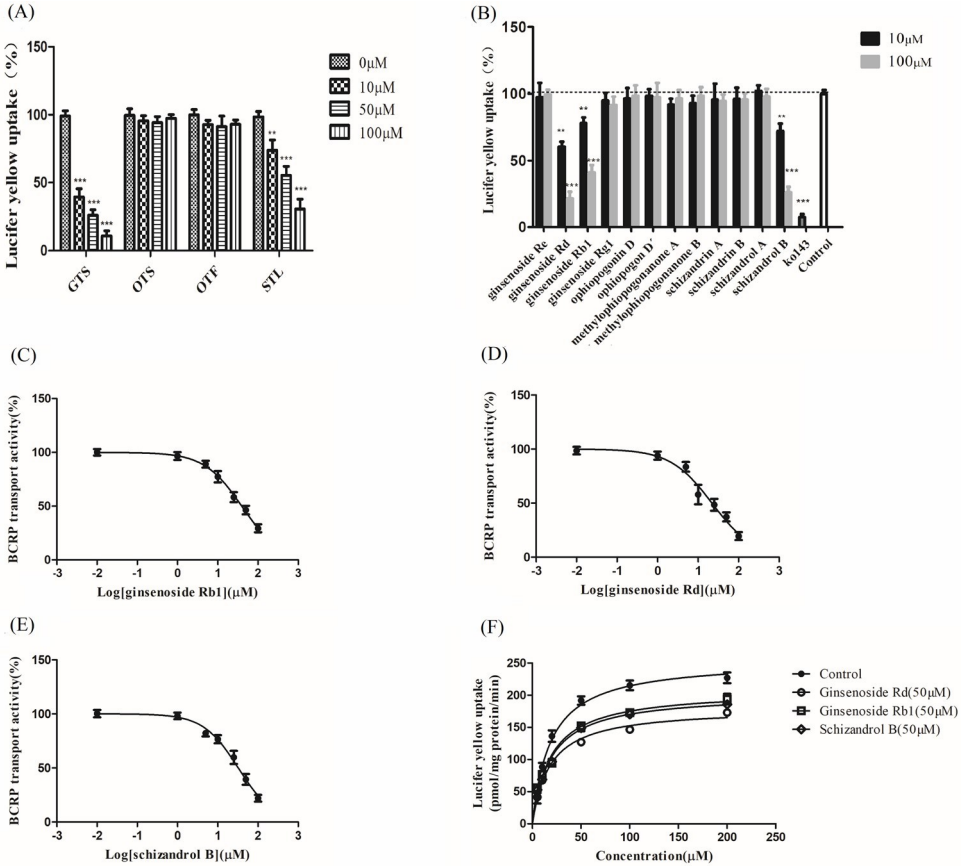


Fig. 7. The uptake of lucifer yellow in the presence of 4 effective fractions (A) and 12 bioactive components of SMF (B) were evaluated in BCRP membrane vesicles. The concentration-dependent inhibition of BCRP by ginsenoside Rb1 (C), ginsenoside Rd (D), and schizandrol B (E) and the inhibition mode (F) were also assessed in BCRP membrane vesicles. The data are presented as the mean ±SE of three independent experiments.

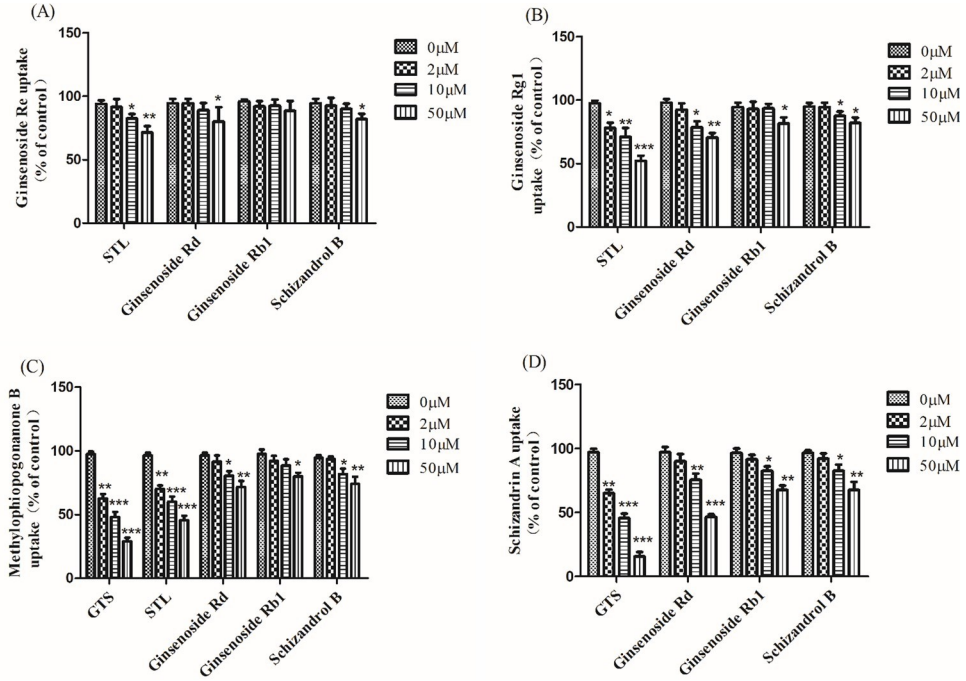


Fig. 8. The effects of BCRP inhibitors in SMF (2, 10, 50 μM) on the uptake of ginsenoside Re (A), ginsenoside Rg1 (B), methylophiopogonanone B (C), and schizandrin A (D) (10 μM) were evaluated in BCRP membrane vesicles. The data are expressed as the mean ±SE of three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

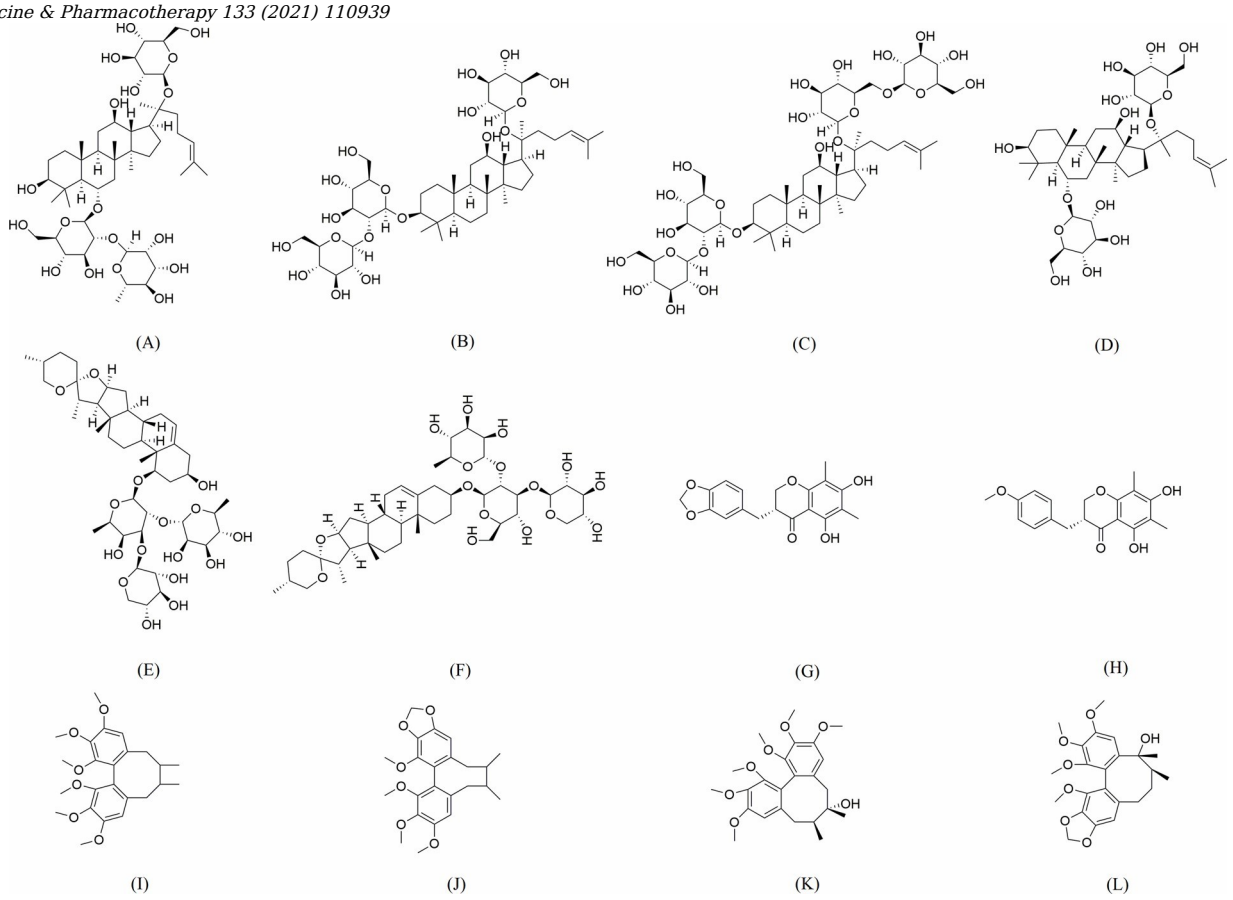


Fig. 9. The chemical structures of ginsenoside Re (A), ginsenoside Rd (B), ginsenoside Rb1 (C) and ginsenoside Rg1 (D), ophiopogonin D (E) and ophiopogonin D´(F), methylophiopogonanone A (G) and methylophiopogonanone B (H), schizandrin A (I) and schizandrin B (J), schizandrol A (K) and schizandrol B (L).

components for BCRP. Therefore, we conclude that there are potential interactions among these active components in SMF at the cellular level that are mediated by BCRP.

To further confirm the potential interactions and compatibility mechanism of complex components in SMF that are mediated by BCRP, the BCRP vesicles were used in the subsequent study. The reliability of the model was verified before the experiment was started, and the kinetic parameters of the classical substrate lucifer yellow in the vesicle were obtained. The 12 bioactive components of SMF were screened for the potential BCRP substrates at two concentrations. The results showed that ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A were the potential substrates of BCRP. Schizandrin A (K_m =37.19 ±6.512 μM) showed the highest affinity to BCRP, followed by methylophiopogonanone B and ginsenoside Rg1 and Re (Fig. 6). The uptake of schizandrin A in LLC-PK1/BCRP cells was significantly decreased than LLC-PK1 cells, and Ko143 showed the strongest inhibition on schizandrin A uptake in LLC-PK1/BCRP cells. Both the cell and vesicle experiments demonstrated that schizandrin A was a high-affinity substrate of BCRP. Subsequently, the inhibitory effects of the 4 effective fractions and 12 bioactive components of SMF were studied to verify which components were potential BCRP inhibitors. The results showed that 100 μM of GTS, STL, ginsenoside Rd and Rb1, and schizandrol B can inhibit the transport of lucifer yellow as mediated by BCRP. And ginsenoside Rd exhibited the strongest inhibitory effect with an IC_{50} of 20.46 ±1.47 μM. The inhibition of ginsenoside Rd and Rb1 and schizandrol B on lucifer yellow transport occurred in non-competitive manner. As earlier stated, the obtained BCRP substrates of ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A and the inhibitor of GTS, STL, ginsenoside Rd and Rb1, and schizandrol B were matched in pairs across BCRP membrane vesicles. GTS, ginsenoside Rd and Rb1, and schizandrol B significantly inhibited the transport of methylophiopogonanone B and schizandrin A. STL, ginsenoside Rd, and schizandrol B exhibited a significant inhibitory effect on the transport of ginsenoside Re, whereas ginsenoside Rb1 did not. STL, ginsenoside Rd and Rb1, and schizandrol B significantly inhibited the transport of Rg1 (Fig. 8). These results implied that there are potential interactions among these active components in SMF that are mediated by BCRP at the vesicular level.

By comparing the results obtained from cellular and vesicular studies, we found an interesting phenomenon that GTS, STL, and ginsenoside Rd and Rb1 are the potential inhibitors of BCRP in vesicles but not in LLC-PK1/BCRP cells. Two factors could explain this observation. First, the LLC-PK1 cells with upregulated BCRP expression are more similar to the localization of normal physiological conditions. This monolayer is tightly connected and can simulate various membrane barriers that are suitable for transport research [25]. For the vesicle model, due to the directionality from inside to outside, the substrates can be directly transported into the vesicles. Thus, the vesicle model is commonly used to study interactions between drugs and efflux transporters [26]. Compared to a single vesicle model, cells are more complex and integrated with multiple receptors, metabolic enzymes,

and transporters. Second, the classic substrates of BCRP used for the experiments are different (MTX for cells, lucifer yellow for vesicles). These differential results obtained in this study can be explained by substrate selectivity of the transporters, which was also previously observed in OATP1B1 [27]. It is also possible that BCRP may have multiple active sites. Thus, the choice of these two experimental models may impact the results of drug transport experiments.

Moreover, the development of efflux transporter inhibitors is relevant to the reversal tumor multidrug resistance [28]. In addition to the components identified in this study, which components have a stronger inhibitory effect on BCRP in SMF? Is it possible to use these components in the clinic? These questions should be answered in future investigations. Based on the limitations of cell testing and the difference between *in vivo* and *in vitro* results, we will soon conduct animal studies to further confirm the potential interactions that are mediated by BCRP. **5. Conclusions**

In summary, GTS, STL, ginsenoside Rd and Rb1, and schizandrol B are the potential inhibitors of BCRP. Ginsenoside Re and Rg1, methylophiopogonanone B, and schizandrin A are the potential substrates of BCRP, and their transport as mediated by BCRP may be inhibited by potential inhibitors in SMF. These major effective components of SMF undergo potential interactions that are mediated by BCRP at the cellular and vesicular levels. The interplay of these bioactive components based on BCRP may be an important compatibility mechanism in SMF.

Contributions of authors

Chunhua Xia, Jinfang Hu and Yuqing Xiong participated in research design. Lingna Wu, Jianming Liu, Jinxia Hou, Tao Zhan and Li Yuan conducted the experiments and performed data analysis. Chunhua Xia, Lingna Wu, Jianming Liu, and Fanglan Liu contributed to the writing of the manuscript.

Declaration of Competing Interest

All authors declare no known conflicts of interest associated with this publication.

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